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Research Article

Multi-regression models to describe some effective parameters in the acute toxicity of silver nanoparticles on the *Artemia franciscana*

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Abstract

The increasing use of consumer products containing nanomaterials (NMs) or utilizing nanotechnology has raised concerns about potential environmental risks associated with NMs. The toxicity of silver nanoparticles (AgNPs) in saltwater microcrustaceans, specifically *Artemia franciscana*, was investigated in this study. This study focused on the key factors of AgNPs concentration and exposure time. To evaluate toxicity, instar I *Artemia* nauplii were exposed to various concentrations of AgNPs (ranging from 0 to 10 mg/L) following the ISO/TS 20787 guideline. Immobilization rates were recorded at 12, 24, and 48 h post-exposure, and the Probit test was used for the statistical analysis. The results indicated significant toxicity to *Artemia* with an EC_{50} value of 4.18 mg/L after 48 hours. Significant relationships were found between immobility and the variables (exposure time and AgNPs concentration) through the use of multiple regression analysis for immobilization. The model explained a high percentage of immobilization variation (R-squared value: 97.74%). The study emphasizes the importance of 'exposure time' and 'concentration' in determining toxicity. The interaction effect between the parameters (concentration and exposure time) was significant. AgNPs concentration had a greater impact on increasing Artemia immobility compared to the exposure time. However, whether the same ranking applies to chronic toxicity or other organisms besides Artemia is uncertain. Understanding the relationship between exposure to NPs and their toxicity is crucial for the safe development of nanotechnology. Future research should address these questions to provide further insights and enable environmentally responsible risk assessments of AgNPs.

Introduction

The use of nanoparticles (NPs) is steadily increasing worldwide, but their environmental impacts, particularly on aquatic organisms, remain largely unknown (Benn and Westerhoff., 2008). Silver element is one of the most widely used NPs with numerous desirable applications due to its antimicrobial properties (Farkas *et al*., 2011). Although nanotechnology offers many positive benefits to the environment and aquaculture (*e.g.* findings of Ziaei-nejad *et al*., 2020; 2021a; 2021b), it also has the potential for adverse effects (Chen and Schluesener, 2008), affecting various aspects of human life.

Toxicological studies have reported the effects of silver nanoparticles (AgNPs) on several marine organisms, including brine shrimp (*Artemia salina*) (Arulvasu *et al*., 2014; An *et al*., 2019; Demarchi *et al*., 2020), Mediterranean mussel (*Mytilus galloprovincialis*) (Bouallegui *et al*., 2018), Copepod (*Amphiascus tenuiremis*) (Sikder *et al*., 2018), white leg shrimp (*Litopenaeus vannamei*) (Lam *et al*., 2020), and brine shrimp (*Artemia parthenogenetica*) (Do *et al*., 2023). In this context, several parameters play a role in the toxicity of NPs to Artemia (Asadi Dokht Lish *et al*., 2019). Among them, the concentration of NPs and the exposure time are considered to be the most important factors (Arulvasu *et al*., 2014; Vijayan *et al*., 2014; An *et al*., 2019). However, it has not been determined which of these parameters has a greater impact on the toxicity of NPs. Therefore, this study aimed to determine the contribution of the two main parameters, namely concentration and exposure time, on the toxicity of NPs using modeling and statistical methods.

Artemia is a microcrustacean with a non-selective filter-feeding property that consumes particles smaller than 50 microns. Due to its crucial role in providing nutrition during the early life stages of aquatic animals, the absence of *Artemia* as live feed would make larviculture impossible for many aquatic species. Additionally, enriched *Artemia* is utilized as a source of food supplements, vitamins, and pharmaceuticals (Sharma *et al*., 2009). Moreover, *Artemia* serves as a bioindicator in highly saline waters. *Artemia franciscana* is a non-indigenous and invasive species reported in Iran, specifically from Maharlo Lake and Nog Lake (Rodgers, 2006). This study aims to investigate the acute toxicity of AgNPs on *A. franciscana* at different concentrations and exposure times. Furthermore, it seeks to develop a regression model to assess the impact of these two parameters on the toxicity of this emerging contaminant (EC).

Materials and methods

Silver nanoparticles and characterizations

Colloidal silver nanoparticles, known as type L (L2000) and commercially available under the name "Nanocid," were supplied by Nano Nasb Pars Co. (Tehran, Iran). According to the manufacturer, this product is a water-based colloid containing spherical silver nanoparticles with a concentration of 4000 mg/L and an average size of 16.6 nanometers. The comprehensive specifications of this colloidal product have been subject to detailed analysis and previously documented in studies by Asghari *et al*. (2012), Johari *et al*. (2013), and Johari *et al*. (2015).

Toxicity experiments

To obtain *Artemia* nauplii for the experiment, the cysts (Inve, Belgium) were hatched according to the method described by Sorgeloos *et al*. (2001). Hatching was performed at pH 7.8, temperature 27°C, and light intensity 2000 lux. Artificial saline water comprised of tap water and 30 g of sea salt was used as the hatching medium (Sorgeloos *et al*., 2001).

The toxicity of AgNPs on *Artemia franciscana* nauplii was assessed according to ISO/TS 20787 (ISO, 2017) (Asadi Dokht Lish *et al*., 2019). Newly hatched nauplii (instar I) were exposed to AgNPs at eight different concentrations (0, 0.2, 1, 2, 4, 6, 8, and 10 mg/L) in glass Petri dishes, with each dish containing 20 nauplii. Each concentration was replicated in triplicate. Artemia immobility was recorded at 12, 24, and 48 h (Rahnama *et al*., 2018).

Statistical and data analysis

Immobilization data were analyzed using Probit Analysis software (version 2.0) to calculate effective concentration values (EC_{10}, EC_{50}, and EC_{90}) (Asadi Dokht Lish *et al*., 2019). The normality of the data was assessed using a Normal Probability Plot. The Contour Plot diagram illustrating the relationship between artemia immobility, exposure time, and AgNPs concentration was generated using Minitab statistical software (version 21). The Poisson Regression Analysis in Minitab software (version 21) was used to obtain the regression model between the parameters. Finally, the model and the parameters involved were checked using multiple regression from various aspects such as the significance of the relationship between the parameters at the 95% level, the percentage of the variables explained by the model, the incremental impact of variables, and the model building sequence.

Results

The results of the toxicity assessment of AgNPs on *A. franciscana* instar I nauplii are presented in Table 1 and Figures 2 to 4. Concentrations causing 10%, 50%, and 90% immobilization in Artemia after 12, 24, and 48 hours of exposure to AgNPs (mg/L) have been shown in Table 1. The EC_{50} after 48 hours of exposure to NPs was 4.18 mg/L. As seen, all effective concentrations at 48 hours show a significant reduction compared to 12 and 24 hours (Table 1). However, overlay the results indicated that immobility in each of the three time intervals increased by increasing in NPs concentration. That is to say, less concentration of AgNPs is needed to reach 50 percent immobilization by a longer exposure period. It means that an EC_{50} value in the first 12 h of the experiment always was higher than EC_{50} at 24h and 48 h.

Table 1: Concentrations (mg/L) causing 10%, 50%, and 90% immobilization of instar I nauplii of *Artemia franciscana* after 12, 24 and 48 hours of exposure to AgNPs. (EC: effective concentration).

ECs	Time (hours)		
	12	24	48
EC_{10}	3.12	2.69	1.30
EC_{50}	8.65	7.25	4.18
EC_{90}	20.89	19.48	13.44

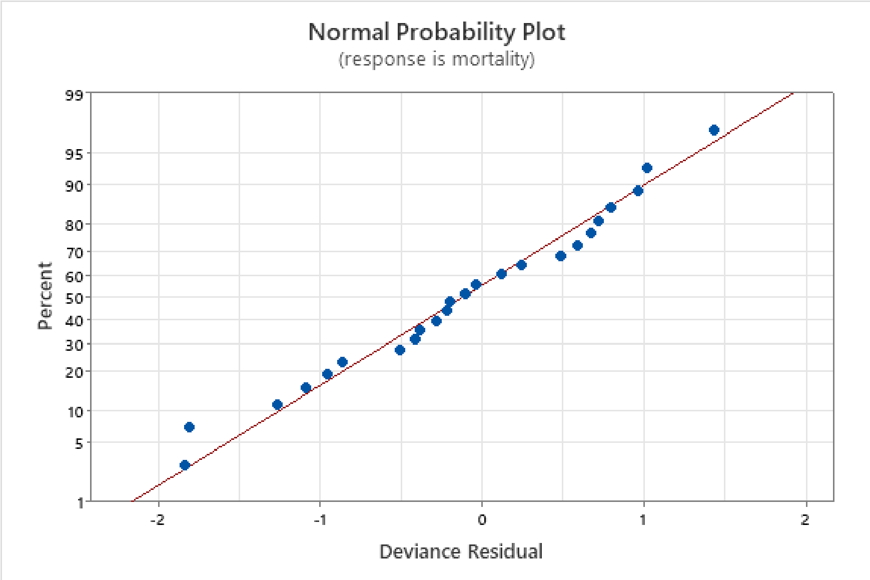

Figure 1: Normal Probability Plot (response is immobility) of variables (exposure time and AgNPs concentration).

The normal probability plot (Fig. 1) indicates that the majority of the research data align closely with the normal probability line (red line), suggesting that the data follow a normal distribution. The regression model and the contour plot (Fig. 2) demonstrate a positive correlation between Artemia immobilization and both exposure time and AgNPs concentration. Artemia immobility increased as the exposure time and AgNPs concentration increased.

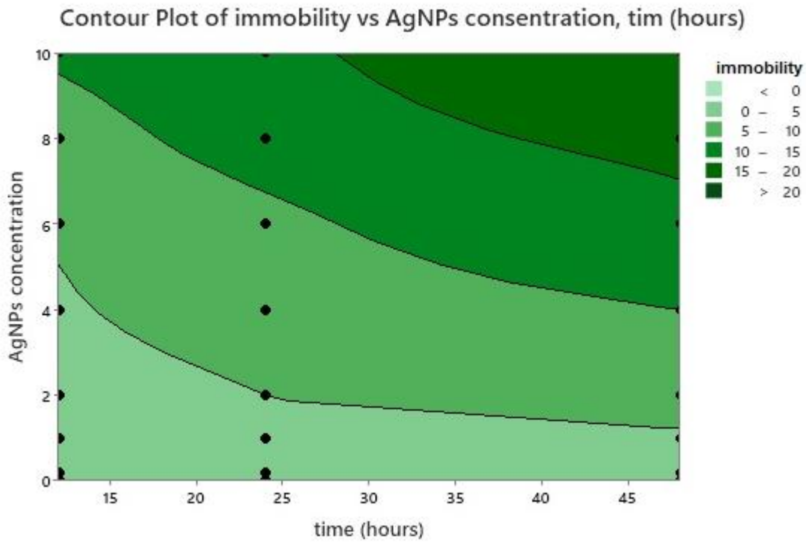

Figure 2: Contour Plot of *A. franciscana* immobility vs exposure time and AgNPs concentration.

The summary report of multiple regression for immobility reveals a significant relationship between immobilization rate and the variables "exposure time" and "AgNPs concentration". Furthermore, the model explains a high percentage of the variation, as indicated by an R-squared value of 97.74%. This suggests that 97.74%

of the variation in immobilization can be explained by the regression model. The final *P*-value, less than 0.001, indicates a statistically significant relationship between the parameters and Artemia immobilization (Fig. 3).

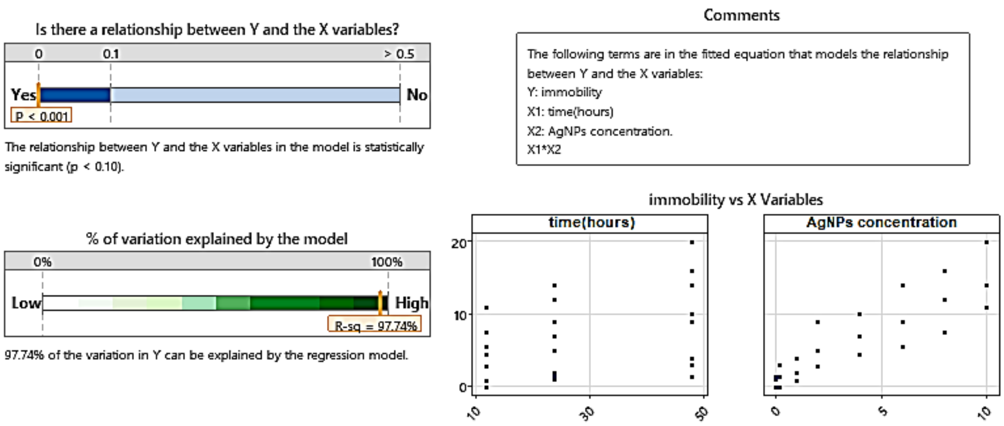

Figure 3: Summary report of multiple regression for Artemia immobilization rate vs exposure time and AgNPs concentration.

According to the regression analysis results, the following model explains the Artemia immobilization in different AgNPs concentrations and at different times:

$$Y = 0.224 + 0.1939 * X1 + 0.02223 * X2$$

Where, Y is the immobilization rate, X1 is AgNPs concentration, and X2 is exposure time (hours).

The model highlights that the interaction effect between exposure time and AgNPs concentration is significant, but the AgNPs concentration factor has a greater impact on increasing artemia immobilization.

Figure 4 illustrates the contribution of each parameter. The incremental impact of variables (Fig. 4, right-hand graph) indicates that AgNPs concentration (represented by the longer bar) provides the most significant information to the model, contributing up to 80.12%. In contrast, the contribution of exposure time (hours) amounts to 21.46%.

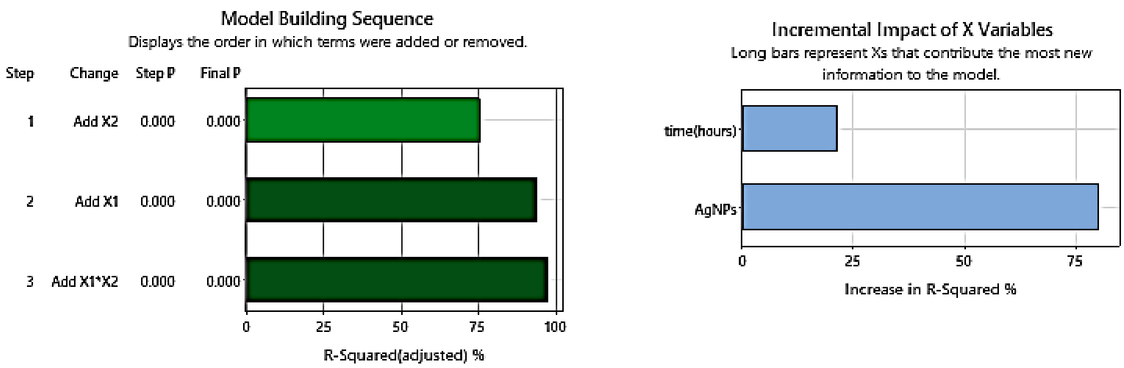

Figure 4: Model Building Report of multiple regression for Artemia immobilization rate vs exposure time and AgNPs concentration (X1: AgNPs concentration, X2: exposure time (hours)). Incremental Impact of Variables (Right Hand) and Model Building Sequence (Left Hand).

Model Building Sequence (Fig. 4, left-hand graph) illustrates the order in which terms were added or removed based on adjusted R-squared. Adjusted R-squared is used when comparing models with different numbers of predictors. The graph indicates that in the first stage when the "exposure time" variable is included alone, the adjusted R-squared exceeds 75.40%. In the second stage, when the model solely includes the "concentration of AgNPs" variable, the adjusted R-squared increases to over 93.73%. Finally, in the third stage, when both variables are incorporated into the model together, the adjusted R-squared further improves to 97.39%. These results demonstrate that both "exposure time" and "concentration of AgNPs" contribute to the model's performance and improve it.

Discussion

The findings of this study provide important insights into the acute toxicity of silver nanoparticles on brine shrimp (*Artemia franciscana*). The EC_{50} value obtained after 48 hours of exposure to AgNPs was determined to be 4.18 mg/L, indicating that the colloid of AgNPs can be classified as a toxic substance for this aquatic species. According to European Union regulations (EC, 1999), substances with EC_{50} values falling between 1 and 10 mg/L are considered toxic and can have adverse effects on aquatic ecosystems.

Comparing our findings with the previous study (Zhao and Wang, 2012), it can be inferred that AgNPs exhibit lower toxicity in saltwater environments than in freshwater. Artemia, serving as an indicator species for highly saline waters, exhibits greater resilience to AgNPs in comparison to freshwater aquatic organisms. The presence of high ionic attraction forces, particularly with chlorine ions, in saltwater significantly mitigates the toxicity of silver ions (Hogstrand and Wood, 1998). Numerous studies have reported the reduction of silver ion toxicity with increasing salinity (Ferguson and Hogstrand, 1998). In saltwater environments, the concentration of chlorine ions naturally rises, leading to the formation of silver chloride compounds, such as silver chloride (Webb and Wood, 2000). These compounds precipitate and are thereby removed from the reach of aquatic organisms inhabiting the water column. Conversely, in freshwater environments, the low concentration of chlorine ions prevents the formation of silver chloride compounds. Consequently, if silver ions enter freshwater, they persist as free ions within the water column, representing the most toxic form of silver that can cause severe toxicity in aquatic organisms. In the case of freshwater fish, silver ions disrupt the activity of the Na^+/K^+ATPase enzyme and reduce the activity of the carbonic anhydrase enzyme in the gills, impeding the absorption of chloride and sodium ions through the gills and leading to blood acidification (Klaine *et al.*, 2008).

Silver nanoparticles are renowned for their antibacterial effects, and their potential application in aquatic health has been investigated. Consequently, it is crucial to ascertain the lethal/effective/inhibitory concentrations and maximum permissible concentration of these substances in different aquatic species, including Artemia, which serves as a primary food source for fish larvae.

Toxicological studies have been conducted to evaluate the impacts of silver nanoparticles on various marine organisms, such as the Mediterranean mussel (*Mytilus galloprovincialis*) (Bouallegui *et al*., 2018), Copepoda (*Amphiascus tenuiremis*) (Sikder *et al*., 2018), white leg shrimp (*Litopenaeus vannamei*) (Lam *et al*., 2020), and *Artemia* (Kumar *et al*., 2012; Vijayan *et al*., 2014; Arulvasu *et al*., 2014; An *et al*., 2019; Do *et al*., 2023). Moreover, several studies have highlighted the significant role of concentration and exposure time in determining the toxicity of AgNPs. For instance, Arulvasu *et al*., (2014) and Demarchi *et al*., (2020) investigated the toxicity of AgNPs in *Artemia salina* and observed increased immobility with higher concentrations and longer exposure times to the NPs. However, to date, there has been a lack of scientific investigations determining the relative significance of various parameters in inducing the toxicity of AgNPs. In fact, it is necessary to study which parameter is more influential in the toxicity of these substances.

Our study's findings indicate that Artemia immobilization rate increased with higher concentrations and longer durations of exposure to AgNPs. Through the analysis of regression model indices, we have established that the model fits the data well and can be utilized to predict Artemia immobility for specific values of exposure time and concentration of AgNPs. This study suggests that concentration plays a stronger role in the acute toxicity of AgNPs on Artemia compared to the exposure duration. Nonetheless, it remains uncertain whether the same ranking applies to chronic toxicity or other organisms beyond artemia. Future research endeavors should focus on addressing these questions to provide deeper insights into the toxicity mechanisms of silver nanoparticles.

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Conflicts of interest

The author declares no conflict of interest.

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